

# Pyridyloxobutyl DNA Adducts Inhibit the Repair of *O*<sup>6</sup>-Methylguanine<sup>1</sup>

Lisa A. Peterson,<sup>2</sup> Xiao-Keng Liu, and Stephen S. Hecht

Division of Chemical Carcinogenesis, American Health Foundation, Valhalla, New York 10595

## ABSTRACT

The carcinogenic tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), both methylates and pyridyloxobutylates DNA. *O*<sup>6</sup>-Methylguanine (*O*<sup>6</sup>-mG) persistence has been correlated to NNK-induced lung tumor formation in A/J mice. The pyridyloxobutylation pathway enhances the tumorigenicity of the methylation pathway. In this paper we test the hypothesis that DNA pyridyloxobutylation increases *O*<sup>6</sup>-mG persistence by inhibiting the repair protein *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase (AGT). Pyridyloxobutylated DNA was generated by reacting calf thymus DNA with the model pyridyloxobutylating agent 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) in the presence of esterase. The alkylated DNA inhibited the ability of partially purified rat liver AGT to repair *O*<sup>6</sup>-mG when it was incubated with AGT prior to the addition of <sup>3</sup>H-methylated DNA. The extent of inhibition was dependent on the amount of NNKOAc reacted with DNA. The ability of NNKOAc-treated DNA to inhibit AGT was destroyed when the DNA was subjected to neutral thermal hydrolysis. Approximately 1 pmol of AGT was inhibited for every 25 to 50 pmol of 4-hydroxy-1-(3-pyridyl)-1-butanone-releasing adducts present in NNKOAc-treated DNA. The inhibitory activity of this alkylated DNA was relatively stable under physiological conditions (pH 7.4, 37°C). Only 13% of the AGT reactive activity was lost after 7 days. When pyridyloxobutylated DNA was incubated simultaneously with <sup>3</sup>H-methylated DNA and AGT, a significant reduction in [<sup>3</sup>H]methyl transfer to AGT was observed. The levels of reduction were similar to those observed when unlabeled methylated DNA containing comparable levels of *O*<sup>6</sup>-mG was substituted for NNKOAc-treated DNA. Based on these results, a cocarcinogenic role for pyridyloxobutylation in NNK-induced lung tumorigenesis is proposed in which pyridyloxobutyl DNA adduct(s) compete with *O*<sup>6</sup>-mG for reaction with AGT resulting in sustained levels of *O*<sup>6</sup>-mG. These enhanced levels then increase the probability of tumor induction by NNK.

## INTRODUCTION

The tobacco-specific nitrosamine, NNK,<sup>1</sup> is an asymmetrical nitrosamine. Therefore, it is activated to DNA-reactive species via two different  $\alpha$ -hydroxylation pathways (Fig. 1). Methyl hydroxylation generates 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide. This reactive intermediate pyridyloxobutylates DNA (1, 2). The structures of these pyridyloxobutyl adducts are unknown. While stable in intact DNA for several weeks both *in vivo* and *in vitro* (3), these adducts decompose to HPB under acid, neutral thermal, or enzyme hydrolysis conditions (1). Methylene hydroxylation leads to methanediazohydroxide formation and subsequent DNA methylation, resulting in the production of 7-mG, *O*<sup>6</sup>-mG, and *O*<sup>6</sup>-mT in tissues of animals exposed to NNK (2, 4).

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<sup>2</sup> To whom requests for reprints should be addressed.

<sup>3</sup> The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 7-mG, 7-methylguanine; *O*<sup>6</sup>-mG, *O*<sup>6</sup>-methylguanine; *O*<sup>6</sup>-mT, *O*<sup>6</sup>-methylthymine; NNKOAc, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; AMMN, acetoxymethylmethylnitrosamine; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; AGT, *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase; MNU, methylnitrosourea; [<sup>3</sup>H]MeDNA, <sup>3</sup>H-methylated DNA; DTT, dithiothreitol; SCX, strong cation exchange; DMN, dimethylnitrosamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; TCA, trichloroacetic acid; TDEG buffer, 50 mM Tris-HCl, 5 mM DTT, 0.1 mM EDTA, 5% glycerol, TDEG buffer, 20 mM Tris (pH 7.4), 5 mM DTT, 1 mM EDTA, 5% glycerol, 5 mM phenylmethylsulfonyl fluoride.

The relative importance of these two pathways in lung tumor induction by NNK was investigated in A/J mice (2). In these studies, the majority of the tumorigenic activity was associated with DNA methylation, specifically with formation and persistence of *O*<sup>6</sup>-mG. The pyridyloxobutylation pathway, studied with the model compound NNKOAc (Fig. 1), was only weakly tumorigenic in mouse lung. However, NNKOAc synergistically increased the tumorigenic activity of the methylating agent, AMMN (Fig. 1), when both compounds were given simultaneously. This enhancement was attributed to the ability of NNKOAc to increase *O*<sup>6</sup>-mG levels. Lung tumor multiplicity was strongly correlated to levels of *O*<sup>6</sup>-mG present 96 h after treatment with NNK, AMMN, or AMMN plus NNKOAc.

One mechanism by which NNKOAc may increase levels of *O*<sup>6</sup>-mG in lung DNA is through inhibition of repair of this promutagenic base. *O*<sup>6</sup>-mG is repaired by AGT via transfer of the methyl group from the *O*<sup>6</sup> position to a cysteinyl residue at AGT's active site (5). This reaction renders the protein inactive, resulting in depletion of cellular AGT levels. Once AGT levels are depleted, *O*<sup>6</sup>-mG accumulates, and the potential for tumor initiation by this adduct increases. Therefore, if the pyridyloxobutyl adducts in DNA compete with *O*<sup>6</sup>-mG for reaction with AGT, an increase in levels of *O*<sup>6</sup>-mG is expected. This would be followed by a corresponding increase in tumor initiation.

The studies described in this paper investigate the ability of the pyridyloxobutylation pathway to inhibit *O*<sup>6</sup>-mG repair by rat liver AGT. The model pyridyloxobutylating compound, NNKOAc, was used to study this question. Upon esterase-catalyzed hydrolysis, it generates the same DNA-reactive pyridyloxobutylating diazohydroxide obtained when the methyl group of NNK is oxidized by cytochrome P-450 (Fig. 1) (3, 6). NNKOAc-treated DNA was used to explore the ability of pyridyloxobutyl DNA adducts to inhibit rat liver AGT activity.

## MATERIALS AND METHODS

**Chemicals.** NNKOAc (7), [<sup>3</sup>H]NNKOAc (6), HPB (7), and 1-(3-pyridyl)-but-2-ene-1-one (8) were prepared as previously reported. Calf thymus DNA and porcine liver esterase were purchased from Sigma Chemical Co. (St. Louis, MO). [<sup>3</sup>H]MNU was obtained from Dupont NEN (Boston, MA, specific activity, 1.7 Ci/mmol).

**AGT Preparation.** Bacterial AGT was a gift from Dr. T. Spratt, American Health Foundation. The *ada* protein was purified from *Escherichia coli* BS21 as previously described (9). The mammalian AGT preparations used in these studies were obtained from livers of partially hepatectomized male Sprague-Dawley rats (Taconic Laboratory, Germantown, NY) using previously published methods (10, 11). Forty-eight h following partial hepatectomy, perfused livers were removed and homogenized in ice-cold TDEG buffer containing 0.5 M NaCl (3 ml/g of tissue). After centrifugation at 150,000 × g for 70 min at 4°C, the supernatant was removed, and ammonium sulfate was added to a final concentration of 1 M ammonium sulfate. The precipitate was removed by centrifugation (20,000 × g, 20 min at 4°C).

In initial studies, the supernatant was made 60% in ammonium sulfate, and the precipitate was collected by centrifugation for 15 min at 10,000 × g, 4°C. The precipitate was then dissolved in TDEG buffer (3% the volume of the original homogenate) and dialyzed overnight against the same buffer. After centrifugation at 23,000 × g for 30 min at 4°C, the supernatant was used in the described studies.

In the studies performed with alkylated DNA, AGT had been subjected to further purification. The 1 M ammonium sulfate supernatant was applied to a phenyl Sepharose column (phenyl Sepharose CL-4B; Pharmacia LKB, Upp-

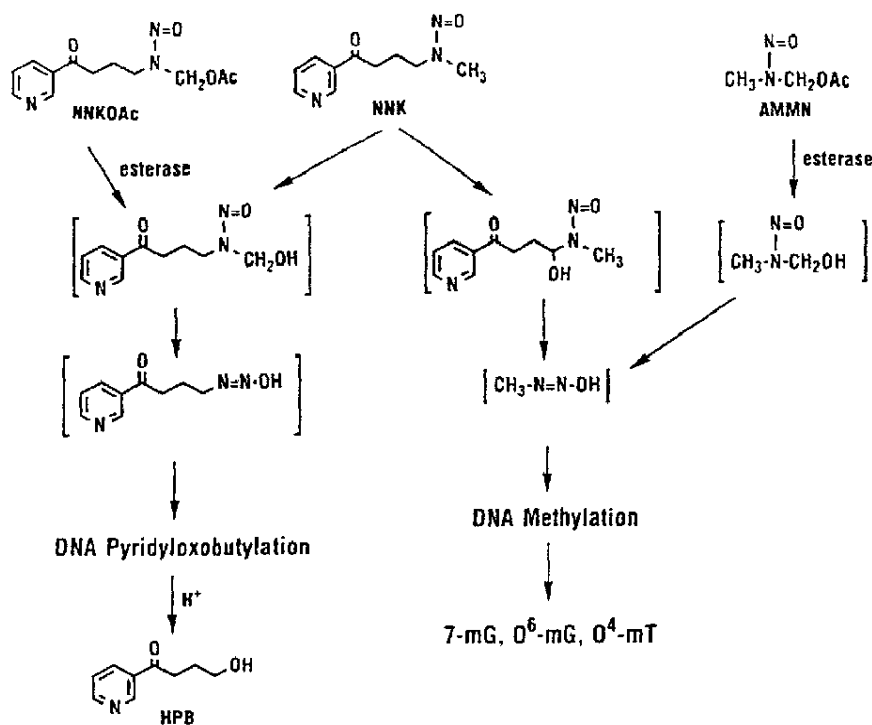


Fig. 1. Activation pathways of NNK, NNKOAc, and AMMN.

sala, Sweden; 10 cm x 16 mm) equilibrated with TDEG buffer containing 1 M ammonium sulfate. The column was eluted with TDEG buffer with a linear gradient from 1 to 0 M ammonium sulfate over 60 min. Active fractions were combined, dialyzed against TDEG buffer overnight, and stored at  $-80^{\circ}\text{C}$  until used. Activity of this fraction remained stable for at least 1 mo. The protein concentration was determined using Coomassie Plus protein assay reagent (Pierce, Rockford, IL).

**Alkylated DNA.** Calf thymus DNA (2 mg/ml) was incubated with NNKOAc (0 to 5 mM), AMMN (0.01 mM), formaldehyde (5 mM), 1-(3-pyridyl)but-2-ene-1-one (5 mM), or HPB (5 mM) and porcine liver esterase (4 units/ml) in 15 mM sodium citrate buffer, pH 7, at room temperature. After 1 h, 2 M NaCl was added (final concentration, 0.33 M), and the DNA was precipitated with ethanol, washed extensively with ethanol, and dried under a stream of  $N_2$ .

Levels of HPB-releasing adducts present in 1.5 and 2.5 mM NNKOAc-treated DNA were estimated by reacting calf thymus DNA with [ $^3\text{H}$ ]NNKOAc under identical reaction conditions. HPB-releasing adduct levels were determined as previously described (1). Briefly, strong acid hydrolysates (0.8 N HCl, 6 h,  $80^{\circ}\text{C}$ ) were neutralized, spiked with unlabeled HPB, and separated on a  $C_{18}$  reverse-phase column (Phenomenex Bondacilone 10, 300 x 3.9 mm) with Solvents A (20 mM sodium phosphate buffer, pH 7) and B (95% methanol:5%  $H_2O$ ) using a linear gradient from 100% Solvent A to 65% Solvent A over 60 min (flow, 1 ml/min). The levels of HPB were determined from the radioactivity that coeluted with the standard, using a Flo-one/Beta radioflow detector (Radiomatics Instruments, Tampa, FL). Guanine concentrations in the hydrolysates were determined by HPLC analysis (4). The levels of HPB-releasing adducts in the 1.5 mM and 2.5 mM NNKOAc-treated DNA were 329 and 807 pmol/ $\mu\text{mol}$  of guanine, respectively.

Levels of O<sup>6</sup>-mG present in 0.01 mM AMMN-treated DNA were determined as previously described (4). Briefly, 0.1 N HCl hydrolysates ( $80^{\circ}\text{C}$ , 30 min) were analyzed by SCX HPLC linked to fluorescence detection. This DNA contained 36.5 pmol of O<sup>6</sup>-mG/ $\mu\text{mol}$  of guanine.

**[ $^3\text{H}$ ]MeDNA.** [ $^3\text{H}$ ]MeDNA was prepared using a modification of published procedures (12). Briefly, [ $^3\text{H}$ ]MNU (1 mCi) was incubated with calf thymus

DNA (25 mg) in 50 mM sodium cacodylate buffer, pH 6, containing 1 mM EDTA at room temperature (total volume, 5 ml). After 18 h, the solution was heated at  $80^{\circ}\text{C}$  for 1 h and cooled. Then, 2 M NaCl (2 ml) was added, and the partially apurinic DNA was precipitated with ice-cold ethanol. The DNA was washed several times with ice-cold ethanol and dissolved in 50 mM HEPES buffer, pH 7.8, containing 1 mM EDTA (10 ml). After dialysis overnight against the same buffer, the DNA was stored in 1-ml aliquots at  $-20^{\circ}\text{C}$ .

O<sup>6</sup>-mG levels were determined by analyzing 0.1 N HCl hydrolysates ( $80^{\circ}\text{C}$ , 30 min) by SCX HPLC linked to a Flo-one/Beta radioflow detector (4). The hydrolysates were eluted using Solvent A (0.02 M ammonium formate (pH 4):6% methanol) and Solvent B (0.2 M ammonium formate (pH 4):8% methanol) with a linear gradient from 100% Solvent A to 80% Solvent A over 30 min (flow, 1 ml/min). Guanine concentrations were determined by HPLC analysis (4), and the amount of O<sup>6</sup>-mG was expressed as pmol of O<sup>6</sup>-mG/ $\mu\text{mol}$  of guanine.

**Effects of Modifiers on AGT Activity.** Initial studies were conducted by incubating NNKOAc (0 to 5 mM), formaldehyde (5 mM), 1-(3-pyridyl)but-2-ene-1-one (5 mM), or HPB (5 mM) with rat liver homogenate containing AGT (0.5 to 1 mg of protein, 0.4 to 0.6 pmol of AGT), porcine liver esterase (2 units), and [ $^3\text{H}$ ]MeDNA (40 to 75  $\mu\text{g}$ , 1 to 2 pmol of O<sup>6</sup>-mG) in 50 mM HEPES buffer (pH 7.8):1 mM DDT, 1 mM EDTA for 30 min at  $37^{\circ}\text{C}$  (total volume, 1 ml).

The ability of alkylated DNA to react with rat liver AGT was determined by incubating alkylated DNA (50  $\mu\text{g}$ ) with partially purified rat liver AGT (0.4 to 0.6 pmol) for 30 min at  $37^{\circ}\text{C}$  prior to the addition of [ $^3\text{H}$ ]MeDNA. Then, the incubations were continued for an additional 30 min. In the studies performed with various amounts of 1.5 or 2.5 mM NNKOAc-treated DNA (0 to 50  $\mu\text{g}$ ), untreated calf thymus DNA was used to bring the total amount of DNA to 50  $\mu\text{g}$ . Blanks in which bovine serum albumin was substituted for AGT were run in each experiment. All experiments were run in triplicate.

AGT activity was determined by measuring the amount of [ $^3\text{H}$ ]methyl transferred to the protein using a modification of published procedures (13). Briefly, the incubations were stopped by precipitation of protein and DNA upon addition of 50% TCA (200  $\mu\text{l}$ ). The mixture was heated at  $80^{\circ}\text{C}$  for 30

min to release purines (including unreacted O<sup>6</sup>-mG) from DNA. The precipitate, containing apurinic DNA and [<sup>3</sup>H]-methylated AGT, was pelleted by centrifugation for 30 min. The supernatant was removed, and the pellet was washed twice with cold 5% TCA. The pellet was dissolved in 0.3 ml of 0.1 N NaOH and transferred to a scintillation vial. After combination with 0.3 ml of H<sub>2</sub>O and 0.6 ml of 0.2 M Tris-HCl washes, scintillation fluid (Picofluor; Packard, Meriden, CT) was added (10 ml), and the amount of [<sup>3</sup>H]methyl transferred to the protein was determined by scintillation counting.

**Adduct Stability Studies.** Untreated DNA or DNA that had been treated with 1.5 mM NNKOAc was dissolved in 100 mM sodium phosphate buffer, pH 7.4 (0.26 mg of DNA/ml), and incubated at 37°C. At 0, 2, 24, 48, 96, and 168 h, an aliquot (50 µg) was removed and incubated with AGT (0.62 to 0.75 pmol) 30 min prior to the addition of [<sup>3</sup>H]MeDNA (1.5 pmol of O<sup>6</sup>-mG; 38 µg of DNA; total volume, 1 ml). AGT activity was measured as described above. The inhibitory activity of NNKOAc-treated DNA was determined by subtracting the AGT activity in the presence of NNKOAc-treated DNA from that observed in the presence of untreated DNA.

**Competition Studies.** Various amounts of 2.5 mM NNKOAc- or 0.01 mM AMMN-treated DNA (0 to 50 µg) and [<sup>3</sup>H]MeDNA (1.5 pmol of O<sup>6</sup>-mG) were incubated with AGT (0.8 pmol) for 30 min at 37°C (total volume, 1 ml). Control calf thymus DNA was added to the incubations so that each reaction mixture contained 50 µg. The amount of [<sup>3</sup>H]methyl transferred was determined as described above.

## RESULTS

Initially, 5 mM NNKOAc was coincubated with esterase, rat liver homogenate, and [<sup>3</sup>H]MeDNA substrate. NNKOAc inhibited the transfer of [<sup>3</sup>H]methyl to AGT in the homogenates by 60%. The inhibition was substantially reduced when esterase was excluded from the incubation mixture. The inhibitory activity was not observed when NNKOAc was replaced with its hydrolysis products, 5 mM formaldehyde, 5 mM HPB, or 1.5 mM 1-(3-pyridyl)but-2-en-1-one. These results demonstrate that NNKOAc interacts with AGT via a reactive intermediate, either directly or through prior formation of a DNA adduct.

NNKOAc can react with DNA to form pyridyloxobutyl adducts (3, 6). The above experiments suggested that these adducts may inactivate AGT. Therefore, the ability of NNKOAc-treated DNA to inhibit AGT activity was investigated. Pyridyloxobutylated DNA was generated by reacting various concentrations of NNKOAc (0 to 5 mM) with calf thymus DNA in the presence of esterase. After isolation, the alkylated DNA was incubated with partially purified rat liver AGT for 30 min prior to addition of [<sup>3</sup>H]MeDNA. This alkylated DNA was capable of inhibiting [<sup>3</sup>H]methyl transfer to rat liver AGT (Fig. 2). The degree of inactivation was dependent on the NNKOAc concentration used to alkylate DNA. In contrast, 5 mM NNKOAc-treated DNA (50 µg) was incapable of inhibiting bacterial AGT (0.4 pmol; total volume, 1 ml). When rat liver AGT was incubated with DNA that had been reacted with NNKOAc hydrolysis products (7), little or no inhibition was

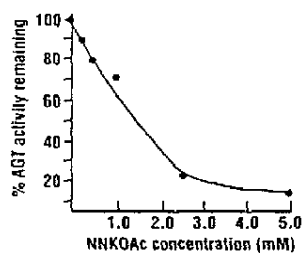


Fig. 2. Inhibition of AGT by NNKOAc-treated DNA. DNA was reacted with the indicated concentration of NNKOAc and esterase and then isolated. The alkylated DNA (50 µg) was incubated with AGT (0.63 pmol) for 30 min prior to addition of [<sup>3</sup>H]MeDNA. The error in each data point is less than or equal to 8%.

Table 1. Inhibitory activity of DNA treated with NNKOAc or its hydrolysis products. Fifty µg of DNA were incubated with AGT (0.4 to 0.6 pmol) 30 min prior to the addition of [<sup>3</sup>H]MeDNA.

Treatment <sup>a</sup>	Relative AGT Activity (%)
Control DNA	100
5 mM NNKOAc-treated DNA	5
5 mM NNKOAc-treated DNA, absence of esterase	90
5 mM NNKOAc-treated DNA, heated <sup>b</sup>	84
5 mM formaldehyde-treated DNA	115
5 mM 1-(3-pyridyl)but-2-en-1-one-treated DNA	94
5 mM HPB-treated DNA	95

<sup>a</sup> DNA was reacted with the indicated compounds in the presence of esterase.

<sup>b</sup> DNA was heated at 100°C for 30 min in 50 mM HEPES buffer, pH 7 containing 1 mM EDTA prior to reaction with AGT.

Table 2. Stability of the AGT-inhibitory activity of NNKOAc-treated DNA. For various times before incubation with AGT (0.62 to 0.75 pmol), 1.5 mM NNKOAc-treated DNA (50 µg) was incubated at 37°C.

Time (h)	pmol of AGT inhibited
0	0.67 ± 0.04 <sup>a</sup>
2	0.65 ± 0.03
24	0.68 ± 0.07
48	0.60 ± 0.05
96	0.58 ± 0.05
168	0.58 ± 0.01

<sup>a</sup> Mean ± SD.

observed (Table 1). The inhibitory activity of NNKOAc-treated DNA was lost when it was subjected to neutral thermal hydrolysis (30 min, 100°C). This treatment releases pyridyloxobutyl DNA adducts from DNA as HPB (1). Therefore, a HPB-releasing pyridyloxobutyl adduct is probably responsible for the inhibitory activity.

Pyridyloxobutyl DNA adducts decompose to HPB in a triphasic manner in pH 7.4 buffer at 37°C with half-lives of 6 h, 5 days, and 18 days (3). In order to determine the stability of the inhibitory activity of NNKOAc-treated DNA, 1.5 mM NNKOAc-treated DNA was incubated in 100 mM phosphate buffer (pH 7.4) at 37°C for various times prior to reaction with AGT. This DNA lost only 13% of its inhibitory activity over 7 days (Table 2).

Since AGT is a single turnover protein, 1 pmol of adduct is expected to inactivate 1 pmol of AGT. It is unclear whether pyridyloxobutylation of DNA results in the formation of a single adduct or multiple adducts. Therefore, it was of interest to determine the ratio of pmol of AGT inactivated to pmol of HPB-releasing adducts in DNA. AGT (0.6 pmol) was incubated with various amounts of 1.5 mM or 2.5 mM NNKOAc-treated DNA (0 to 50 µg) prior to the addition of [<sup>3</sup>H]MeDNA. The total amount of DNA present in the incubations was held constant (50 µg) with the balance made up by untreated calf thymus DNA. Levels of HPB-releasing adducts present in the NNKOAc-treated DNA were estimated by reacting calf thymus DNA with [<sup>3</sup>H]NNKOAc under identical reaction conditions. A plot of pmol of AGT inactivated versus pmol of HPB-releasing adducts present in the DNA (Fig. 3) indicates that approximately 1 pmol of AGT is inhibited for every 25 to 50 pmol of HPB-releasing adducts. This result suggests that 2 to 4% of HPB-releasing adducts appear to be responsible for AGT inactivation. While this value was derived from estimated adduct levels, it is a reasonable approximation according to our experience with the alkylation reaction and the known stability of these adducts (3).

A comparable result was obtained when various amounts of partially purified rat liver AGT (0.1 to 0.6 pmol) were incubated with 20 µg of 2.5 mM NNKOAc-treated DNA containing 11 pmol of HPB-releasing adducts. In these incubations, the maximum amount of AGT reacting with pyridyloxobutylated DNA was 0.22 ± 0.03 pmol (*n* =

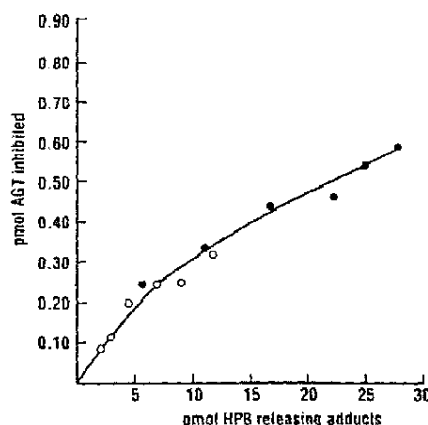


Fig 3 Dependence of AGT inhibition on levels of HPB-releasing adducts in DNA. Various amounts of 1.5 mM and 2.5 mM NNKOAc-treated DNA (0 to 50  $\mu$ g) were incubated with AGT (0.6 pmol) for 30 min prior to addition of [ $^3$ H]MeDNA ( $\circ$ , 1.5 mM NNKOAc-treated DNA;  $\bullet$ , 2.5 mM NNKOAc-treated DNA). The error in each data point is less than or equal to 8%.

5), suggesting that approximately 2% of the HPB-releasing adducts were responsible for reaction with AGT.

In the experiments described above, NNKOAc-treated DNA had been incubated with rat liver AGT for 30 min prior to addition of [ $^3$ H]MeDNA. However, methyl and pyridyloxobutyl DNA adducts are present at the same time in NNK-treated animals. Therefore, it was of interest to determine what effect NNKOAc-treated DNA had on [ $^3$ H]-methyl transfer when this DNA was incubated simultaneously with methylated DNA and AGT. Various amounts of 2.5 mM NNKOAc-treated DNA (0 to 50  $\mu$ g) and [ $^3$ H]MeDNA, containing 1.5 pmol of  $O^6$ -[ $^3$ H]mG, were incubated with AGT for 30 min, and the amount of [ $^3$ H]methyl transferred to the protein was determined. The results shown in Table 3 indicate that the level of [ $^3$ H]methyl transferred to AGT is diminished by the presence of HPB-releasing adducts. In addition, the extent of inhibition of [ $^3$ H]methyl transfer caused by the AGT-reactive pyridyloxobutyl adducts in NNKOAc-treated DNA was comparable to that observed with similar levels of  $O^6$ -mG (Table 3).

## DISCUSSION

In these studies we have demonstrated that NNKOAc reacts with DNA to generate an adduct that inhibits the repair of  $O^6$ -mG by rat liver AGT activity. These results are consistent with the hypothesis that pyridyloxobutylation is capable of enhancing the initiating activity of  $O^6$ -mG by decreasing its repair by AGT.

The inactivation of rat liver AGT by NNKOAc-treated DNA was attributed to a HPB-releasing pyridyloxobutyl DNA adduct. None of the NNKOAc's hydrolysis products reacted with DNA to form a product that was capable of inactivating AGT. In addition, esterase was required to generate NNKOAc-treated DNA that reacted with AGT. Furthermore, the inhibitory activity of NNKOAc-treated DNA could be removed from DNA by neutral thermal hydrolysis, a condition known to release HPB from pyridyloxobutylated DNA (1).

Attempts to characterize pyridyloxobutyl adducts have been unsuccessful as a result of their instability under chemical and enzymatic hydrolysis conditions (1, 14). Therefore, it is not known how many different types of adducts are formed upon DNA pyridyloxobutylation. It is likely that several adducts are formed based on the multiphasic decomposition of HPB-releasing DNA adducts under physiological conditions (3). The observation that 1 pmol of rat liver AGT is inhibited for every 25 to 50 pmol of HPB-releasing adducts present in NNKOAc-treated DNA suggests that only 2 to 4% of the thermally labile HPB-releasing adducts are capable of inhibiting AGT. While this percentage is based on estimated adduct levels, it is a reasonable approximation based on our knowledge of the alkylation reaction and adduct stability (3). These results indicate that a specific adduct may be responsible for the inhibition. An analogous situation is known to exist for DNA methylation. In this case, the specific AGT-reactive adduct,  $O^6$ -mG, represents 7% of overall DNA methylation by MNM (15). Therefore, a likely candidate for the AGT-reactive pyridyloxobutyl adduct is an  $O^6$ -pyridyloxobutylguanine derivative. If an  $O^6$ -pyridyloxobutylguanine is responsible for the inactivation of AGT by NNKOAc-treated DNA, it is possible that the bulky alkyl group is transferred to the active-site cysteine of AGT.

The ability of NNKOAc-treated DNA to react with rat liver AGT but not the *E. coli* ada protein is consistent with previously reported studies on the kinetics of  $O^6$ -alkylguanine repair by these two proteins. The mammalian protein has broader substrate specificity for reaction with  $O^6$ -alkylguanine derivatives than bacterial AGT (16, 17). For example,  $O^6$ -benzylguanine reacts rapidly with rat liver AGT but not with the ada protein (17).

These pyridyloxobutyl adducts appear to compete effectively with  $O^6$ -mG for reaction with AGT. If an  $O^6$ -pyridyloxobutyl adduct is responsible for the inhibition of AGT, then these results are somewhat surprising. In general, the ability of AGT to react with  $O^6$ -alkylguanine residues decreases as the alkyl group becomes larger (16, 18). Studies using rat liver AGT have established that the relative rate of repair of  $O^6$ -alkylguanine residues in DNA is methyl > ethyl, *n*-propyl > *n*-butyl > isopropyl, isobutyl > 2-hydroxyethyl (16, 18). An apparent exception to this rule is the free base  $O^6$ -benzylguanine, which is a better substrate for mammalian AGT than  $O^6$ -mG (17, 19).

Table 3 Competition of NNKOAc- or AMMN-treated DNA with [ $^3$ H]MeDNA for reaction with AGT

Various amounts of 2.5 mM NNKOAc- or 0.01 mM AMMN-treated DNA (0 to 50  $\mu$ g) and [ $^3$ H]MeDNA containing 1.5 pmol of [ $^3$ H] $O^6$ -mG were incubated with AGT for 30 min at 37°C (total volume, 1 ml).

pmol of HPB-releasing adducts in treated DNA	pmol of $O^6$ -mG in treated DNA	pmol of AGT-inhibitory adducts <sup>a</sup>	pmol of [ $^3$ H]methyl transferred to AGT	% of inhibition of transfer
0		0	0.47 $\pm$ 0.04 <sup>b</sup>	0
5.5		0.17	0.46 $\pm$ 0.02	2
11.0		0.33	0.36 $\pm$ 0.02	23
16.5		0.49	0.33 $\pm$ 0.01	30
22.0		0.66	0.29 $\pm$ 0.001	38
27.4		0.82	0.21 $\pm$ 0.01	55
	0.25	0.25	0.41 $\pm$ 0.05	13
	0.50	0.50	0.34 $\pm$ 0.03	28
	0.74	0.74	0.22 $\pm$ 0.01	53
	0.99	0.99	0.18 $\pm$ 0.02	62

<sup>a</sup> Inhibitory HPB-releasing adducts were calculated as 3% of the total HPB-releasing adducts.  $O^6$ -mG is the inhibitory adduct in AMMN-treated DNA.

<sup>b</sup> Mean  $\pm$  SD

The benzyl group is believed to overcome its steric hindrance through delocalization of the positive charge occurring during the displacement reaction (19). Therefore the ability of an *O*<sup>6</sup>-alkylguanine derivative to react with AGT parallels its reactivity with nucleophiles.

In the case of an *O*<sup>6</sup>-pyridyloxobutylguanine derivative, the transfer reaction may be facilitated by the neighboring group participation of the carbonyl oxygen. Cyclic oxonium ion intermediates have been detected in alkylation reactions by pyridyloxobutylating agents (7, 20). The possible participation of a cyclic oxonium intermediate might also explain the instability of this adduct under neutral thermal hydrolysis conditions.

While a mechanism involving the transfer of a pyridyloxobutyl group to the active-site cysteine is a probable mechanism for the inactivation of AGT by NNKOAc-treated DNA, we cannot exclude other mechanisms at this time. The potential role of an *O*<sup>6</sup>-pyridyloxobutylguanine adduct in the inhibition of AGT by NNKOAc-treated DNA and the mechanism of inhibition are currently under investigation.

The ability of the pyridyloxobutylation pathway to inhibit AGT *in vivo* suggests a possible role for this reaction in NNK-induced tumorigenesis. The effect of the pyridyloxobutylation pathway on AGT activity *in vivo* has not been studied. AGT levels are depleted in Clara cells in the lungs of NNK-treated rats (21). *O*<sup>6</sup>-mG persists in these cells for up to 8 days after NNK treatment (10 mg/kg/day for 4 days), whereas it is efficiently removed from other lung cell types (21). While NNK-induced rat lung tumors appear to arise primarily from Type II cells, a strong correlation between tumor formation and *O*<sup>6</sup>-mG levels in Clara cells has been observed (22). Similarly, *O*<sup>6</sup>-mG persists for at least 2 wk in NNK-treated A/J mouse lungs, and there is also a strong correlation between *O*<sup>6</sup>-mG levels at 96 h and lung tumor formation in this species (2). The effect of NNK on AGT levels in A/J mouse lung has not been investigated.

The role the pyridyloxobutyl DNA adducts play in the depletion of AGT levels and persistence of *O*<sup>6</sup>-mG in NNK-treated animals is not known. However, there are some suggestions that pyridyloxobutylation may be involved. First, *O*<sup>6</sup>-mG levels in lungs of mice treated with NNKOAc and AMMN were higher than those in lungs of mice treated with AMMN alone (2). In addition, *O*<sup>6</sup>-mG in lung DNA formed upon treatment of A/J mice with 10  $\mu$ mol of NNK persisted longer than that from treatment with 0.5  $\mu$ mol of AMMN despite the similarity in adduct levels at 24 h (2). The absence of the pyridyloxobutylation pathway in the AMMN-treated animals could explain the more rapid disappearance of *O*<sup>6</sup>-mG in these animals.

Devereux *et al.* (23) reported that, in rat lung, the methylation efficiency of NNK was dramatically increased at the lower doses of NNK. A similar increase was not observed with DMN. While this difference could be caused by differences in cytochrome P-450 enzyme forms involved in the activation of NNK and DMN, another contributing factor may be the presence of the pyridyloxobutylation pathway.

The results of these studies may also have implications in the risk assessment of smokers exposed to NNK. Since the two activation pathways appear to be catalyzed by different P-450 enzymes in mouse and rat microsomes (24, 25), there could exist a polymorphism in humans for these two pathways. Two smokers that have the same level of P-450 responsible for activation of NNK to a methylating agent might be expected to have the same risk of NNK-induced lung tumors. However, if one of these smokers lacks the enzyme that catalyzes the pyridyloxobutylation pathway and if that pathway is a cocarcinogen for the methylation pathway, that smoker might be at reduced risk for lung tumor formation. Studies in which the levels of pyridyloxobu-

tylated hemoglobin and DNA adducts in smokers have been measured suggest that a polymorphism for this pathway is possible (26, 27).

In summary, NNKOAc is capable of inhibiting rat liver AGT activity by reacting with AGT directly or via a DNA adduct. The results of this study suggest that this could be a biologically relevant process. Therefore, we propose that the pyridyloxobutylation pathway of NNK metabolism is a "cocarcinogen" for the methylation pathway. The mechanism of cocarcinogenesis involves the generation of pyridyloxobutyl DNA adducts which compete with *O*<sup>6</sup>-mG for reaction with AGT. This competition decreases the amount of *O*<sup>6</sup>-mG repaired and consequently increases the probability of tumor initiation by this promutagenic base.

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